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Use of Radioligand Binding Techniques in Neurotoxicology

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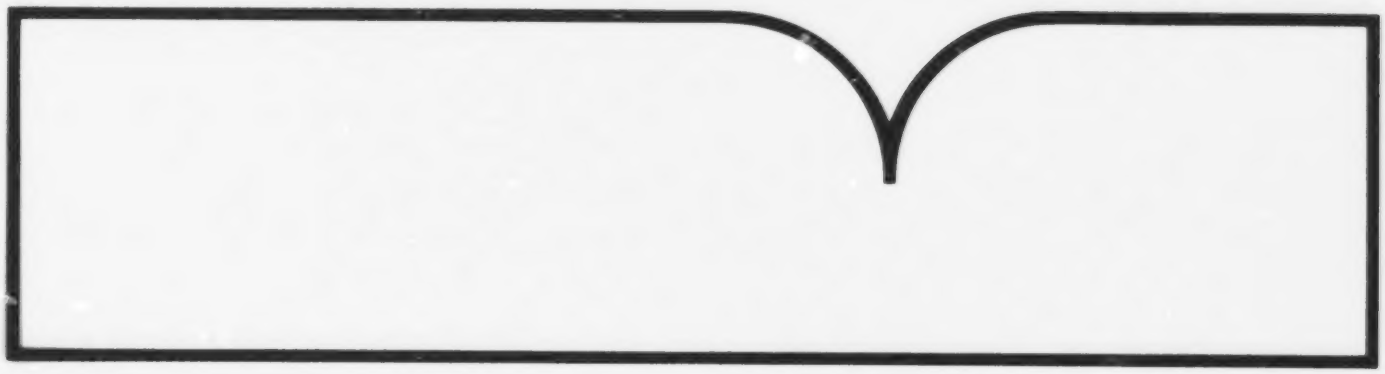

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16. ABSTRACT

Although it had been suggested that chemical transmission was an important physiological mechanism, confirmation awaited Loewi's finding that autonomic nerve impulses resulted in the release of specific chemical stimulants (1). These results led to an inevitable conclusion--that there must be specific recognition sites that can respond to the presence of the minute amounts of these chemical transmitters. In the intervening years, it became clear that the interaction of a ligand (*e.g.*, a chemical transmitter) with a binding site (*e.g.*, a receptor) could be characterized not only physiologically, but also biochemically (2). However, the available biochemical methods required relatively large amounts of tissue containing the receptors, especially considering the low concentration of receptors in the nervous system.

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THE USE OF RADIOLIGAND BINDING TECHNIQUES IN NEUROTOXICOLOGY

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INTRODUCTION

Background

Although it had been suggested that chemical transmission was an important physiological mechanism, confirmation awaited Loewi's finding that autonomic nerve impulses resulted in the release of specific chemical stimulants (1). These results led to an inevitable conclusion--that there must be specific recognition sites that can respond to the presence of the minute amounts of these chemical transmitters. In the intervening years, it became clear that the interaction of a ligand (*e.g.*, a chemical transmitter) with a binding site (*e.g.*, a receptor) could be characterized not only physiologically, but also biochemically (2). However, the available biochemical methods required relatively large amounts of tissue containing the receptors, especially considering the low concentration of receptors in the nervous system.

The availability of radiolabeled chemicals helped resolve this problem, since the ability to detect very small amounts of tracer permitted the study of receptors without their prior purification (3,4). The use of radioligands to identify receptors in crude membrane preparations of central nervous system origin was first successfully applied with opiate systems (5-7). In the intervening years, these techniques have been applied in pharmacological and neurobiological studies to every putative or purported chemical messenger (*i.e.*, neurotransmitter or neuromodulator). In part, the widespread use of these methods results from the relative technical ease by which large quantities of data may be generated. Moreover, radioligand binding assays have provided many answers to previously perplexing questions. For example, the ability of some drugs or toxicants to interact potently with *several* types of receptors has been demonstrated with these procedures. In addition, with appropriate application of radioligand binding the concentration of a certain type of receptor and the affinity of that binding site for the radioligand can be determined.

Why have radioligand techniques received so much attention in recent neurotoxicological studies? One possibility is that a neurotoxicant may bind directly to a receptor, a result that would have immediate consequences *in vivo*. Radioligand techniques can provide a cogent method for studying this type of interaction. Alternately, administration of the toxicant to an animal can cause changes in the concentration or affinity of radioligand binding sites as a result of compensatory responses to the toxicant-induced perturbation of the nervous system. Thus, radioligand techniques have the potential to help answer important toxicological questions.

Definition of Receptors and Binding Sites

Chemical communication between cells in the nervous system necessarily involves the release of a chemical messenger by one cell, and a subsequent recognition of that chemical signal with resulting alterations of cellular function. As an aid to understanding how this process works, an idealized synapse (the communication area between neurons) is illustrated in Figure 1. Of particular relevance to this chapter are the postsynaptic receptors, the ones generally believed to play a major role in intercellular communication. These receptors serve several physiological functions--they may be linked to enzymes (*e.g.*, adenylate cyclase or ATPases), ion channels, or various other transduction systems. It should be noted that the presynaptic cell may also have receptors whose function is thought to involve regulation of the release of neurotransmitter by the presynaptic cell (8).

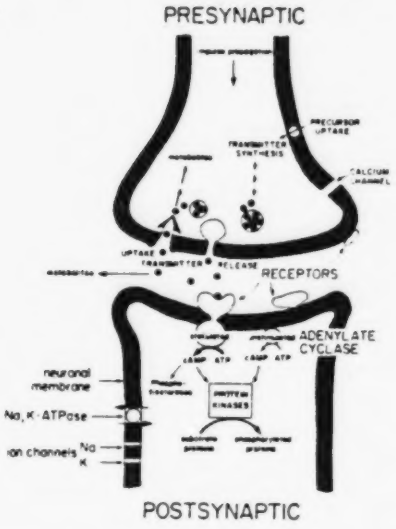

Fig. 1. Schematic drawing of synapse (from Ref. 9). Note that receptors are believed to exist on both presynaptic and postsynaptic cellular elements.

From this scheme, it is clear that even for a single neurotransmitter there may be at least two distinct classes of receptors, the pre- and postsynaptic types. The latter may have additional diversity because it can be linked to a variety of postsynaptic events. Moreover, the anatomy of synaptic connections complicates this situation even further. Synapses occur not only between axonic terminals and dendrites (axo-dendritic), but may also be axo-somatic, axo-axonic, etc. Because the type of synapse may affect the characteristics of the intrinsic receptors, it is clear that receptor multiplicity is a significant confounding factor in attempting to define specific testable hypotheses in neurotoxicology. This will be discussed in later sections on analyzing data.

The receptors themselves are primarily proteinaceous in nature, although associated lipids and carbohydrates are critical to function. Most neurotransmitter receptors are believed to be situated in biological membranes, especially the plasmalemma. This presents difficulties in attempting to use biochemical methods originally developed for soluble systems. (Available techniques for examining membranes have been reviewed in an earlier volume of this series

(9)). As has been mentioned, the function of receptors is to recognize and bind a given chemical substance (*i.e.*, a neurotransmitter or neuromodulator), thus triggering a variety of physiological events including neuronal depolarization, activation of a "second messenger" within the cell (*e.g.*, cyclic AMP), transport of molecular or ionic components within the neuron, synthesis of protein, hormonal release, etc. Drugs may interact with receptors by mimicking neurotransmitter actions (agonists) or by blocking access of the transmitter to the receptor (antagonists). However, radioligand techniques may not always be able to distinguish between pharmacological agonists and antagonists.

Finally, if one posits the existence of a specific receptor, there are two consequences. One is that the receptor should be saturable with ligands specific for it. In addition, there should be specificity in binding some ligands versus others, using as a basis known pharmacological actions of the various compounds being compared. The implications of these statements are the subject of the discussions that follow. Although the focus of this chapter is on receptors for both neurotransmitters and neuromodulators, these techniques have been applied to studies of binding sites of other kinds of endogenous molecules, as well as xenobiotics.

Examples of CNS Receptors

The majority of work dealing with receptors has been with the "classical" neurotransmitters. The study of these led to the development of criteria by which categories of evidence could be evaluated to verify a transmitter role for a candidate compound (10). Among these transmitters are the biogenic amines (*e.g.*, norepinephrine, dopamine and serotonin), acetylcholine, and some amino acids (*e.g.*, γ-aminobutyric acid, glycine, taurine and glutamate). However, research during the last decade has resulted in a proliferation of candidate chemical messengers, and, as a result, reappraisal of the criteria used to evaluate them. More than a dozen peptides are believed now to have roles as neurotransmitter-modulators, although they differ from the "classical" transmitters in many significant ways. This metamorphosis in neurobiology provides a foundation for neurotoxicologists. Although basic developments in the various neurosciences make the neurotoxicologist's task one of continually increasing complexity, inherent to the increasing complexity is the opportunity for better understanding of the mechanisms and consequences of neurotoxicity.

The compound for which a transmitter role was first established was acetylcholine. Cholinergic receptors have been classified into two major subtypes based on pharmacological characteristics; both have been reported in the brain.

Muscarinic cholinergic receptors are predominant in the corpus striatum, cerebral cortex, hippocampus, superior and inferior colliculus, midbrain-pons, thalamus and hypothalamus (11-13). Nicotinic receptors have been localized in cerebellum, thalamus, hypothalamus, cortex, caudate, hippocampus and superior colliculus as well as in tectum and tegmentum, and pons (14-16).

Catecholamines may be the most intensively studied transmitters in the CNS. Receptors for norepinephrine and epinephrine were first divided into two subtypes, α- and β-adrenergic, based on pharmacological responses in the peripheral nervous system (17). Peripheral stimulation of the α-receptor results in vasoconstriction and smooth muscle contraction, while β-receptor stimulation leads to vasodilation, smooth muscle relaxation and influences on cardiac contractility and rate. In brain, α-receptors have been found in cerebellum, pons, frontal cortex and hypothalamus (18) whereas β-receptors have been identified in cortex, caudate and cerebellum, as well as hippocampus and diencephalon (19-20). These subtypes have more recently been further differentiated (*e.g.*, α_1, α_2, β_1, β_2), and the occurrence of the subtypes has been hypothesized to be of physiological significance. Thus, it has been reported that α_2-adrenergic receptors may represent principally presynaptic localization whereas α_1-adrenergic receptors are postsynaptic (21).

Dopamine (DA) receptors are commonly found in terminal fields of the major dopaminergic tracts, which include the nigrostriatal, mesolimbic, and tuberoinfundibular dopaminergic systems (22-23). Thus, high concentrations of DA receptors are found in the striatum, olfactory tubercles, nucleus accumbens, frontal cortex and pituitary (24). Attempts have been made to subdivide further the DA receptor into D_1 and D_2 subtypes (25), the D_1 receptors being those coupled to adenylate cyclase, whereas the D_2 receptors are not (26). Both subtypes may exist in some, but not all, regions (25), although some have suggested these receptor subtypes represent interconverting forms of the same receptor (27-28). Recently, even greater multiplicity of receptors has been proposed (29-31).

Another important biogenic amine is 5-hydroxytryptamine (serotonin). Receptors for serotonin have been identified in the cell fields of serotonergic neurons projecting from the midbrain raphe nuclei. These receptors are present in frontal cortex, hippocampus, striatum, hypothalamus, amygdala, septum, and superior colliculus (32-36). Subtypes of serotonin receptors have also been proposed (37-38). The other major biogenic amine is histamine, and receptors for this compound have been reported in several areas of the CNS (39-40).

Of the amino acids, γ-aminobutyric acid (GABA) has been the most scrutinized. GABA receptors have been reported in cerebellum, cortex, globus pallidus,

olfactory bulb, hippocampus, striatum and hypothalamus (41-45). An interesting issue related to GABA receptors has to do with the class of drugs known as benzodiazepines (*e.g.*, diazepam, chlordiazepoxide, etc.). Receptors for benzodiazepines have been co-localized in the CNS with GABA receptors (46-47), and it has been postulated that the benzodiazepine receptor (for which the endogenous ligand is unknown) is a subunit of the GABA receptor complex (48). Several α-amino acids have also been suggested to be chemical messenger candidates (49-51), and therefore might have possible roles in future neurotoxicological studies. These include excitatory amino acids such as glutamate and aspartate (49) and other inhibitory amino acids like taurine (50) and glycine (51).

The last group of chemical messenger candidates of particular interest is the neuropeptides. Spurred by the identification of hypophyseal releasing factors (52-53), studies during the last decade have demonstrated transmitter-modulator roles for many peptides (54). As discussed in the Introduction, the first application of radioligand techniques to CNS tissue was with opiate systems (5-7), having sites now believed to function as receptors for several opioid (opiate-like) peptides. These receptors are found in many brain areas including limbic system structures (such as the amygdala, thalamus and hippocampus), in cortex, striatum, globus pallidus and nucleus accumbens, and also in stria terminalis, entopeduncular nucleus, hypothalamus, habenula, locus coeruleus, periventricular substance, raphe nucleus, and superior colliculus (55-57).

Although opioid receptors have received the greatest attention, receptors for other neuropeptides are also known. These include those for substance P (58), vasoactive intestinal polypeptide (59), bombesin (60), cholecystokinin (61), thyrotropin releasing hormone (62), calcitonin (63,64), angiotensin II (65), and neurotensin (66). As these receptors become better characterized, and as further studies confirm the role of the endogenous peptides in the CNS, it would seem appropriate that neurotoxicological studies will also be directed toward them.

Use of Direct vs. Indirect Methods for Studying Receptors

Radioligand binding methods may be considered a direct means for the study of receptors, since the assay purportedly measures the interactions of the ligand with the binding site(s) of interest, presumably receptors. As mentioned in the Introduction, appropriate use of these techniques may permit evaluation of the ability of some toxicants to bind directly to receptors, and also permit estimation of toxicant-induced perturbations of receptor systems in the CNS. However, a major strength of the technique, the fact that radioligand methods

use relatively crude, unpurified fractions of heterogeneous tissue, is also an important weakness. It should be reiterated that neurotransmitter receptors and binding sites for radioligands may not always be one and the same. It is possible to detect binding sites that are not representative of actual receptors, and some receptors may not be measured because of unsuspected biochemical variables (*e.g.*, preparation of tissue, type of buffer, location of receptor in tissue, etc.). One way to avoid spurious interpretation of data is to correlate radioligand binding with various indirect measures of receptor function. These can include estimations of neuronal activity (*e.g.*, neurotransmitter synthesis and turnover), physiological studies, or behavioral tests assessing performance of a variety of tasks such as motility, operant responding, pain sensitivity, consummatory behavior, learning and memory, etc. It may be especially valuable to combine these approaches with appropriate pharmacological challenge studies.

Many behavioral paradigms have been studied in sufficient depth such that at least some of the neurochemical substrates have been determined. The effects of prior exposure to a toxicant on the performance of such a behavioral task may give clues as to some toxic sequelae. However, if the action of a drug (known to affect such a task) is either potentiated or attenuated in animals pretreated with a toxicant, valuable data are gained that may be used for correlation with specific receptor changes. Thus, the use of radioligand methods alone to assess receptor function reveals little of how a neurotoxicant affects neuronal function. Changes in binding characteristics can be due to non-specific effects on the receptor-membrane complex, to a presynaptic lesion resulting in compensatory postsynaptic supersensitivity, or to secondary effects arising from changes in other neurotransmitter systems. Moreover, the radioreceptor assay is also sensitive to modifications in methodology or assay conditions and, as such, results must be interpreted with caution. For these reasons, we believe that the use of a multidisciplinary approach is essential to obtain toxicologically relevant information. These other disciplines should not be limited to behavioral techniques, but can involve application of any of the fundamental neurosciences.

METHODOLOGICAL FACTORS

Theory and Experimental Considerations

In this section, we will attempt to provide a general introduction to practical aspects of radioligand binding assays as they relate to toxicological studies. Several excellent reviews or compendia of receptor binding techniques are available, and it is essential that reference be made to these for more

detailed discussion of binding theory and methods prior to actual use (67-71). For neophytes, one of the least expensive and most useful documents is the manual from the Society of Neuroscience Short Course (72).

The methods used to measure radioligand binding are technically simple and rapid, but they can be very sensitive to small procedural variations. Virtually every assay parameter may affect the resulting data. These include the type of tissue and method of preparation, type and strength of buffer, concentration and types of ions, temperature and length of incubation, presence of reducing agents or nucleotides, and filter (or pellet) washing and handling. Not all of these variables have physiological ramifications.

Further, appropriate use of radioligand binding methodology requires a thorough understanding of the interaction of the radioligand with its binding sites in the tissue being studied. This usually requires quantification of the affinity of the radioligand for its binding sites (*i.e.*, the K_D) as well as estimation of the number (concentration) of binding sites (*i.e.*, the B_{max}). The methods that have been applied to problems in neurotoxicology are usually based on equilibrium considerations, but kinetic parameters should also be determined to be sure that similar values are obtained by both types of studies. Novices are urged to consult one of the available treatises (*e.g.*, 67-72) prior to initiating these experiments.

Analysis and Interpretation of Saturation Curves

Use of radioligand binding assays is based on the association of a radiolabeled ligand with a recognition site, hopefully a physiological receptor. Frequently (but not always) this reaction is assumed to be reversible, and can be represented by the following notation:

$$H + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RH \qquad \text{(Equation 1)}$$

where H represents the concentration of unbound hormone, R the concentration of unbound receptor, RH the concentration of the associated hormone-receptor complex, and k_1 the rate of association (units of $M^{-1}\ t^{-1}$) and k_{-1} the rate of dissociation (units of t^{-1}) of the hormone-receptor complex.

By definition, at equilibrium the rates of the forward and reverse reactions are equal. Therefore, Equation 1 can be rewritten as follows:

$$\frac{[H]\,[R]}{[RH]} = \frac{k_{-1}}{k_1} = K_D = \frac{1}{K_A} \qquad \text{(Equation 2)}$$

where K_D is the equilibrium dissociation constant (units = M) and K_A is the equilibrium association constant (units = M^{-1}).

Let us say that B_{max} represents the total number of receptors (*i.e.*, the maximum number of sites that can be specifically labelled), and let B represent the amount of receptor bound with hormone (*i.e.*, RH in Equations 1 and 2). From this, the number of receptors not occupied is:

$$R = B_{max} - B$$

It is then possible to rearrange Equation 2 in a form that allows us to graphically represent the data in a manner similar to the Scatchard plot (2):

$$\frac{[H]\,[R]}{[RH]} = K_D \qquad \text{(Equation 2)}$$

where $R = B_{max} - B$.

Therefore:

$$\frac{B}{H} = \frac{1}{K_D}\,(B_{max} - B)$$

or rearranging

$$\frac{B}{H} = -\frac{1}{K_D}\,B + \frac{B_{max}}{K_D} \qquad \text{(Equation 3)}$$

It has been common practice to express the concentration of free ligand at equilibrium (H in Equations 1-3) as F. In Equation 3, B/H would therefore be equivalent to B/F. If one plots the relationship of B/F (ordinate) versus B (abscissa), one typically obtains a plot such as is shown in Figure 2. This relationship, devised for populations of homogeneous binding sites, describes a situation at equilibrium. Binding experiments must be designed with this consideration in mind if reasonable estimations for B_{max} and K_D are to be obtained.

Data from saturation curves, transformed to form a Scatchard plot (cf. Equation 3 and Figure 2) are usually plotted with F (the concentration of free ligand at equilibrium) being measured by subtracting the total amount of

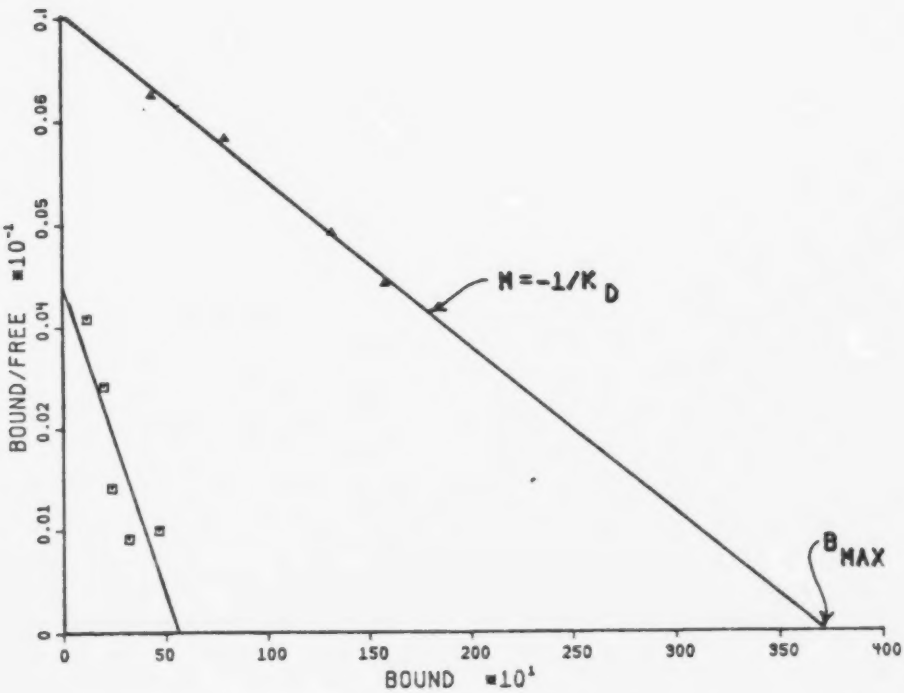

Fig. 2. Saturation isotherm (Scatchard plot) of the binding of ^{3}H-dopamine to membranes from the rat brain striatum (unpublished data from this laboratory). Filled triangles = binding of ^{3}H-dopamine alone (K_D = 15 nM; B_{max} = 197 fmoles/mg protein). Open squares = binding of ^{3}H-dopamine in presence of 0.5 mM ascorbate (K_D = 4 nM; B_{max} = 41.3 fmoles/mg protein). Note that the addition of ascorbate to the incubation buffer markedly decreases both the B_{max} and the K_D. These data illustrate the importance of characterization of any assay, and of the dangers in making conclusions based solely on a preliminary estimate of binding.

radioactivity bound to tissue from the amount of radioactivity added. It is clear that the plot shown in Figure 2 has an intercept on the abscissa (that is, when B/F = 0) of B = B_{max}. Moreover, the slope of the line, from Equation 3, is $-1/K_D$. Thus, this type of analysis allows estimation of the maximal number of binding sites, and an equilibrium dissociation constant. Other factors related to these analyses will be discussed later. As was mentioned earlier, it is also possible to calculate a dissociation constant (K_D) by experimentally determining the two relevant kinetic constants, k_1 and k_{-1} (see Equation 2). The K_Ds determined by each method should be in agreement.

For various reasons, Scatchard plots of saturation data may yield nonlinear curves. Numerous methods have recently been published for analyzing biphasic Scatchard plots by graphical (73-75), and by computer methods (76-77). Analyses and interpretation of nonlinear Scatchard plots can become quite complex, and may represent heterogeneity of binding sites, positive or negative cooperativity, various artifacts (non-equilibrium conditions, metabolism of the radioligand), and other variables (70,77). Moreover, the theoretical applicability of Scatchard analyses has been challenged (78-79).

Whatever the resolution of these issues (78-79), there has been a compelling argument offered that other mathematically equivalent methods of analysis may be of greater use. For example, Keightley and Cressie (80) have shown that for "real" data (*i.e.*, including experimental "outliers"), the Woolf Plot (81-82) is of much greater reliability, especially when coupled with weighted robust regression analysis (80). Preliminary application of these methods in the authors' laboratory has confirmed these conclusions. Thus, care in selecting a method of analysis may increase the power of studies in neurotoxicology. It should be noted that the highest affinity binding site does not necessarily represent the most physiologically relevant site. If multiple sites are present, it is important when possible to determine the effects of toxicants on low affinity, as well as high affinity, specific binding sites. This will aid in correlating changes in receptor parameters with alterations in drug or neurotransmitter sensitivity.

In addition to the question of the existence of multiple binding sites, there is also the issue of whether binding sites are cooperatively linked. Analysis of binding data using Scatchard plots provides some of the needed information. However, the Hill Plot, originally developed to explore the binding of oxygen to hemoglobin, is also commonly used (83). From Equation 2, it is possible to rearrange terms to derive an equation similar in format to the Michaelis-Menten equation, *i.e.*:

$$B = \frac{(B_{max})\ F}{K_d + F}$$

In the more generalized condition where cooperativity may exist, and using n to define the degree of cooperativity or multiplicity, this equation becomes:

$$B = \frac{(B_{max})\ F^n}{K_D + F^n} \qquad \text{(Equation 4)}$$

The Hill Equation is a logarithmic transformation of Equation 4:

$$\log \frac{B}{B_{max} - B} = n \log F - \log K_D \qquad \text{(Hill Equation)}$$

A plot of log $[B/(B_{max} - B)]$ versus log F will permit estimations of n (or more commonly n_H) to be made. However, as can be seen, this plot is affected by both multiplicity of binding sites and the degree of cooperativity between sites. It should be noted that Hill plots can also be used for analysis of displacement curve data (*vide infra*).

Non-specific Binding

Non-specific binding is usually defined as the association of the ligand to tissue, filters, or tubes at sites other than the receptor site under investigation. Because of tacit assumptions made about receptor characteristics (84), non-specific binding usually is considered to be non-saturable and of low affinity. Thus, some radioligand may associate with charged groups on the membrane, dissolve into membrane lipids, become trapped within the pellet, or remain with buffer that adheres to filters. All of these factors contribute to non-specific binding. In addition, radioligands may bind to enzymes, membrane proteins, or similar physiological sites other than the receptors that one wishes to measure.

In practice, saturable specific binding is usually defined as that displacable by an excess of an unlabeled ligand that is pharmacologically, if not chemically, similar to the radioligand. It is evident that binding to "non-specific" sites can easily be confused with binding to the desired receptor. This problem could be circumvented if a compound was available which could selectively displace the radioligand only from one type of site, but highly specific molecules like this are generally not known. Operationally, a low concentration of a compound having a higher affinity for the receptor than for other binding sites is used to define non-specific binding. This concentration is one sufficient to displace the radioligand from the receptor of interest, while having smaller or negligible effects on radioligand bound to other sites. This is illustrated in Figure 3.

When using a displacing compound which is selective for one of multiple binding sites, one must be certain that substances used to generate displacement curves are competing with specific binding, rather than lowering non-specific binding. Thus, when studying the effect of a toxic compound upon receptor binding, the effect of the compound on non-specific binding should

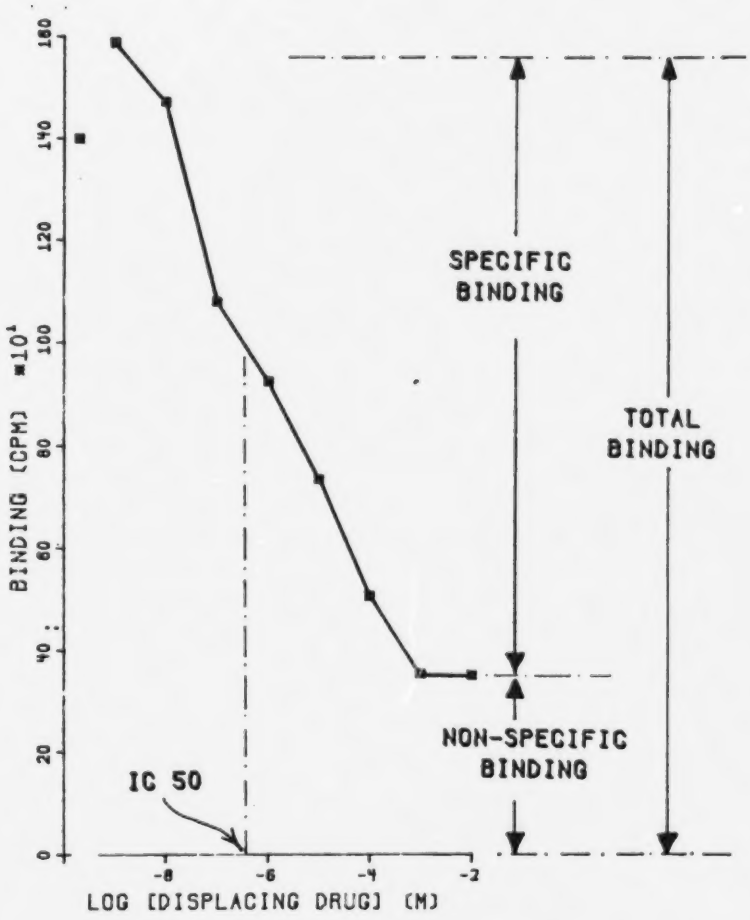

Fig. 3. Displacement of ^{3}H-dopamine (3.4 nM) by varying concentrations of non-radioactive dopamine-like drug. Displacement curves can be used for two purposes. First, the effects of the toxicant itself on the binding of the radioligand can be determined. For example, these data indicate that this drug displaces ^{3}H-dopamine with high potency, suggesting a direct receptor interaction. The estimations of total binding, non-specific and specific binding that are made if this displacing drug were to be assumed to be a specific receptor occupier are also shown.

always be determined. Both the criteria for identifying a binding site as a specific receptor, and problems in identifying non-receptor interactions have been discussed in several reviews (67-72,85).

Care must be taken in selecting the concentration of tissue and ligand for receptor assays. The amount of tissue in the binding is ideally limited to a concentration at which less than 10% of the radioactivity added is bound at equilibrium (86). When studying the displacement of labeled ligand by unlabeled compounds, both the concentration of the labeled ligand and the receptor site are optimally limited to 10% of the dissociation constant of the radioligand (87). Higher receptor concentrations will result in a lower apparent affinity of the ligand for the receptor, as measured either by Scatchard plots of saturation data or by displacement of radioligand by unlabeled compounds. Therefore, when studying the effects of toxic compounds on receptor affinity, the investigator should attempt to use a tissue concentration at which receptor densities in control and treated groups are roughly similar.

Analysis of Displacement Curve Data

The affinity of a compound for a binding site is often calculated from the concentration of it which produces a 50% inhibition of the specific binding of a radioligand, binding to the same sites. The value derived experimentally, the IC_{50} (see Figure 3), can be converted to a more meaningful term, the K_i, by the relationship derived by Cheng and Prusoff (88) for enzyme inhibitors:

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_D}} \qquad \text{(Equation 5)}$$

In this equation, [L] equals the concentration of the radioligand used in the displacement curve, and K_D is the dissociation constant of the radioligand derived from a Scatchard-type analysis (see Figure 2). For this relationship, the ligand concentration should ideally be limited to 10% of the K_D to prevent underestimation of the affinity of displacers. Moreover, K_i's may be dependent on tissue concentration. The apparent K_i obtained from Equation 5 can be converted to a true K_i by defining the apparent K_i at several tissue concentrations. If a plot of apparent K_i vs. tissue is made, the extrapolated value at zero tissue concentration is the true K_i. Since this is very tedious, in comparing the K_i's of compounds for receptors of tissue from treated vs. untreated animals, the concentrations of tissue and labeled ligand should be similar for both control and treated groups.

The Hill plot is also used in the analysis of displacement curve data, again to determine the existence of cooperativity or multiplicity. With displacement curves, the Hill plot involves determining a factor called P, P being the percent of maximum binding that occurs at each concentration of displacing or inhibitory ligand (I). The Hill plot is then the relationship between log [P/(100-P)] versus log I. As with saturation isotherms, the slope of the resulting line is the Hill coefficient, n_H. Moreover, the IC_{50} can be determined more rigorously from the Hill plot since it will be the concentration (I) of displacing ligand where the quantity log [P/(100-P)] is equal to zero, *i.e.*, P = 50%. If a technique such as linear regression is used, the determination of an IC_{50} is less arbitrary than when obtained simply from visual inspection of the data.

Finally, Table 1 provides a list of some of the well studied radioligands. The application of these to neurotoxicological problems is discussed in the following section.

Selection and Preparation of Tissue

The brain area(s) chosen for toxicological studies are selected for one or more of several reasons: (1) because available evidence implicates specific neurotransmitter systems (*e.g.*, toxicant-induced pathological changes in specific neurons, or alterations in neurotransmitter concentrations); (2) because a particular neurotransmitter is believed to be involved in a behavioral or physiological event known to be altered by the toxicant under study; (3) because the region selected has a high density of receptors that are amenable to study. From these facts, decisions about selection of brain regions for dissection and analysis are made. Tissue can be used either fresh or after frozen storage, depending on the receptor being studied. Binding of some radioligands, such as ^{3}H-quinuclidinyl benzilate (QNB), is often unaffected by prior freezing of tissue, while binding of other radioligands, such as ^{3}H-GABA, is reported to be altered by freezing. It is preferable that this issue, as well as other methodological ones, be addressed prior to actual experiments.

It should be clear, from the earlier discussion of receptor multiplicity, that the ideal situation would be if various populations of receptors could be purified quantitatively prior to analysis. This is at present an impossible task since homogeneous receptor preparations even from simple tissues have been accomplished only recently (89-92). From areas of the central nervous system which may contain millions of neurons and even greater numbers of synapses, this ideal will probably never be achieved. Within this limiting framework, there are some experimental modifications that have potential utility.

TABLE 1. EXAMPLES OF SOME NEUROTRANSMITTER RECEPTORS AND LIGANDS PROPOSED FOR THEIR STUDY

Neurotransmitter/ Modulator	Ligand	Agonist	Antagonist	Reference
	Biogenic Amine Receptors			
Acetylcholine				
Nicotinic	^{125}I-α-bungarotoxin	-	yes	15
	^{3}H-nicotine	yes	-	185
Muscarinic	^{3}H-QNB	-	yes	13
Dopamine, Serotonin	^{3}H-spiperone (spiroperidol)	-	yes	33,37
Dopamine	^{3}H-haloperidol	-	yes	27
Dopamine	^{3}H-dopamine	yes	-	27
Dopamine	^{3}H-ADTN	yes	-	186,187
Serotonin	^{3}H-LSD	(mixed)		34
Serotonin S_1	^{3}H-serotonin	yes	-	34
S_2	^{3}H-ketanserin	-	yes	38
Norepinephrine α	^{3}H-WB4101	-	yes	188
Norepinephrine β	^{3}H-dihydroalprenolol	-	yes	189
Norepinephrine β	^{125}I-iodohydroxy-benzylpindolol	-	yes	19
	Peptide Receptors			
Opioid Peptides	^{3}H-etorphine	(mixed)		6
Opioid Peptides	^{3}H-met-enkephalin	yes	-	190
Opioid Peptides	^{3}H-naloxone	-	yes	191
Opioid Peptides	^{125}I-met-enkephalin	yes	-	191
Substance P	^{125}I-substance P	yes	-	58
Angiotensin II	^{125}I-angiotensin II	yes	-	4,65
	Amino Acids and Miscellaneous Compounds			
GABA	^{3}H-GABA	yes	-	44,45
GABA	^{3}H-muscimol	yes	-	42
"Benzodiazepine"-like	^{3}H-flunitrazepam	(? unknown)		46,196
Glycine	^{3}H-strychnine	yes	-	192,193
Taurine	^{3}H-taurine	yes	-	194,195

Brain tissue can be partially purified using differential centrifugation procedures. For example, synaptosomal membranes should have a high density of neurotransmitter receptors, and there are some relatively rapid procedures for obtaining crude synaptic membranes. Thus, modifications of published methods (88,89) might involve the following steps:

1. Fresh brain region is homogenized in 15 volumes of ice cold 0.32 M sucrose with ten strokes of a teflon/glass homogenizer with the pestle rotated at a speed of 850 rpm.
2. The crude homogenate is centrifuged for 10 min at 1000 g to sediment blood cells, nuclei and cell bodies. The resulting supernatant is centrifuged for 20 min at 20,000 g to sediment synaptosomes. The supernatant, containing microsomal membranes, is discarded.
3. The last pellet is resuspended in water using a Polytron (or similar apparatus), thus lysing the synaptosomes. This mixture is centrifuged at 8000 g for 20 min, resulting in a bilayer pellet. The lower, darker layer consists largely of mitochondria, whereas the upper, lighter layer is enriched in synaptosomal membranes. Because it is more loosely sedimented than the mitochondrial layer, it is easily removed along with the supernatant by gently swirling the tube and decanting the contents. Other, somewhat more lengthy procedures can be used to obtain more highly enriched subcellular fractions. These have been reviewed in a recent volume of this series (9).

For toxicological studies, there are two major reasons for using fractions enriched in synaptosomal membranes or other subcellular constituents. One is to reduce the amount of tissue that contains relatively low concentrations of the receptor, thereby reducing non-specific binding of the radioligand and increasing the precision of the assay. A second, related reason is to study selectively regions in which a toxicant is believed to cause greater effects. In the future, subcellular fractionation procedures will be used more extensively to achieve these objectives in neurotoxicological studies.

Another consideration with these methods is to insure that one is accurately assessing binding to cell-surface receptors, rather than other processes that might cause apparently similar effects. These include transport and storage of the ligand or metabolism of the ligand. Homogenization in hypotonic buffer, followed by several washings, has been used to decrease the concentrations of endogenous compounds that bind to the receptor of interest and could inhibit (or augment) the binding of the radiolabeled ligand. Washing of tissue is

accomplished by several cycles of resuspending the membranes in fresh buffer and centrifuging. An additional strategy has sometimes been used to decrease further the content of endogenous ligands. Preincubation of the tissue at 37°C is one way to accelerate the metabolic degradation of endogenous biogenic amines or peptides prior to addition of the radiolabeled ligand. Elimination of interference from endogenous compounds may have particular importance when studying brain tissue of animals treated with toxic compounds, since the toxicants may alter the concentration of neurotransmitters or other compounds that might interfere with binding of the radioligand.

A major experimental issue in neurotoxicological studies is the potential interaction, *in vitro*, of the toxicant with the receptor or the biochemical environs of the receptor. In fact, determining how the toxicant affects radioligand binding *in vitro* is critical to understanding the effects of the toxicant on the receptor system after *in vivo* treatments. As a general rule, in tissue from animals treated with a toxicant, problems in interpretation will occur if the toxicant is present in the membrane preparation at a concentration that will alter the binding of the radioligand. The previous section on displacement curves describes how this may be assessed.

Once the tissue has been suitably prepared, the actual binding assay can be performed. The tissue is incubated in the presence of the radioligand until equilibrium is reached; that is, the rates of association and dissociation of the ligand with the receptor are equal. The time required may vary from less than a minute to an hour or more, and is dependent on the temperature of incubation. After equilibrium is reached, the membranes with associated radioligand must be separated from the solution containing unbound ligand. The two most widely used techniques are vacuum filtration through glass fiber filters and centrifugation. In both instances, the filter or pellet is rinsed with buffer to reduce the amount of non-specifically bound radioligand. When ligands with lower affinities or rapid dissociation rates are being assayed, centrifugation is frequently used since the receptors stay at equilibrium with ligand trapped within the pellet. Since only the membrane-receptor complexes on the surface of the pellet are exposed to the wash (assuming a rapid washing procedure), there is less chance for dissociation of the ligand. Thus, although higher non-specific binding may result from radioligand trapped within the pellet, centrifugation does provide some advantages. Radioligands with higher affinities and/or slower dissociation rates can be used in filtration assays in which the entire preparation is exposed to a rinse for several seconds. Discussion of criteria for utilizing filtration vs. centrifugation, as well as several other practical

aspects of binding methodology, are available in several fine reviews (68-72).

One of the most commonly used apparatus for filtration assays is the filter port available from Millipore Corporation, each of which supports 12 filters. Larger units containing more filter supports are available from several sources. As with incubation tubes for filtration assays, centrifugation assays must be performed in tubes that do not readily bind the radioligand. Frequently polypropylene or similar plastics are used in tubes of sizes 15 ml or smaller. Following rinsing of pellets in centrifugation assays, tissue solubilizers such as Protosol (New England Nuclear) or NCS (Amersham) may be added to dissolve the pellets, thus aiding transfer to counting vials. Centrifugation assays have also been performed in 4 ml polypropylene vials (*e.g.*, Biovials from Beckman or Omnivials from Wheaton). In this case, scintillation cocktail can be added directly to the tubes following digestion of the pellet.

APPLICATION OF RADIOLIGAND BINDING TECHNIQUES TO NEUROTOXICOLOGY

The Lessons of Model Systems

What relevant information can radioligand binding techniques provide for neurotoxicologists? Understanding the answer to this question is essential to prevent misapplication of these methods, or misinterpretation of resulting data. The information provided in the previous sections gives some of the requisite theoretical background related to technical issues. However, a review of how these techniques have been used in basic neurobiological studies may exemplify proper application to neurotoxicology. The methods previously discussed were divided into displacement curves and saturation isotherms. Different types of information may be gleaned from these applications.

Displacement Assays. The most direct use of radioligand techniques is to determine if a toxicant can interact with a receptor directly (possibly activating the receptor or preventing the normal interaction of the receptor with endogenous ligands), or otherwise alter interactions of the binding sites with their ligands. This type of effect can be detected most readily by the use of displacement curves, which can demonstrate if a toxicant will compete with a radioligand for specific binding sites (see Figure 3). This is the most straightforward application of radioligand methods and it is of utility especially when other types of pharmacological and toxicological data have been accumulated. Many highly toxic natural products of plants and animals are known to affect specific receptors; the conclusive link in many of these studies has been the use of radioligand methods.

As examples, many ergot alkaloids are now known to bind with high affinity to putative receptors for dopamine and serotonin (93). The toxicity of several potent marine toxins is known to result from high affinity binding to ion channels and related sites in excitable membranes (94). The acetylcholine receptor is known to be affected by plant and animal constituents [*e.g.*, α-bungarotoxin from snake venom, or various Belladonna alkaloids (94)]. In general, those compounds that are highly potent often have high affinity binding sites on the membranes of excitable cells, since their toxicity usually results from cardiovascular or respiratory effects (94). The use of radioligand methods to study these types of mechanisms is appropriate, and frequently highly rewarding.

To avoid problems in interpretation, cognizance should be given to basic tenets of biochemistry. For example, it has been clearly shown using model compounds that the binding of a "specific" radioligand may be affected by perturbation of the surrounding microenvironment of the receptor (95). This finding is hardly surprising since a cardinal principle of biochemistry is that the environment of a receptor, enzyme or other biological molecule is critical to its function. Moreover, it is often difficult to know if the microenvironment, *in vitro*, bears any relationship to events in the intact organism. For this reason, caution in using displacement methods to assess toxicity is warranted.

The dilemma created by these issues was illustrated by recent studies with the food color Erythrosin B (FD&C Red #3). In 1979, two groups reported that, *in vitro*, Erythrosin B inhibited the neuronal uptake of dopamine and other neurotransmitters (96-97). The uptake process is sodium dependent, and a later study reported that these effects of Erythrosin B on neurotransmitter uptake were a result of decreases in sodium transport--the proximate cause apparently being competition of Erythrosin B with ouabain binding sites (98-99). These experiments employed radioligand methods (utilizing ^{3}H-ouabain) to make these conclusions about the ultimate mechanism of Erythrosin B toxicity. Are the underlying conclusions necessarily valid?

Shortly after the first reports of Erythrosin B inhibition of neurotransmitter uptake, this laboratory suggested that, rather than being specific effects, these results were due to relatively non-specific biochemical interactions. For example, it was found that the *percentage* of inhibition of uptake was dependent not only on Erythrosin B concentration, but also on the tissue concentration used in the *in vitro* experiment (100). In addition, these studies demonstrated that this food color also perturbs three other biochemical systems in a non-specific fashion, one of these being the binding of ^{3}H-spiperone to striatal dopamine receptors (100). Since these findings were consistent with the ability

of Erythrosin B to interact with various classes of lipophilic sites (101), it appeared that caution was required in using any *in vitro* technique alone to assign toxicity to Erythrosin B. It should be noted that Alivisatos *et al.* (102) reported that ouabain-sensitive Na^+, K^+-ATPase activity could be inhibited *or* enhanced by different model *lipophilic* compounds in a manner consistent only with perturbation of the lipid microenvironment. Thus, used alone, data about Erythrosin B effects on ^{3}H-ouabain binding may lead to erroneous conclusions when interpreted *in vacuo*.

In summary, the effects of Erythrosin B on ^{3}H-ouabain binding, as assessed by displacement curves, are certainly real. However, for reasons discussed above, it is unlikely that this observation has neurotoxicological relevance (100,103-105). With Erythrosin B (96) or with other compounds (95), the conditions of the displacement assay may markedly alter the results obtained. In our opinion, caution should be used in extrapolating neurotoxicity only from data indicating that potential toxicants can displace radioligands (106-108).

Saturation Assays: Scatchard and Related Analyses. The most commonly used application of radioligand techniques does not rely on determinations of whether a compound directly interacts with a receptor, but rather on the assumption that neurotoxicants may alter receptor characteristics through two possible distinct mechanisms. One possibility is that a toxicant may cause cell death, and the direct loss of receptors located on these target cells can be detected. Alternately, the assumption is made that many perturbations of the central nervous system cause compensatory (homeostatic) responses that are likely to be expressed by changes in the number or characteristics of receptors for the neurotransmitter in the perturbed pathway. These mechanisms would be expected to cause changes in the number of binding sites and/or affinity of those sites for the radioligand.

What is the basis for believing that these assumptions are valid? There is a large body of fundamental neurobiological information available that can be used to evaluate their validity. The first assumption states that when cells die, their cell surface receptors will be lost. In fact, there is evidence that various lesions of neuronal populations will result in loss of the receptors expected to be on those cells (109-113). It is hardly surprising that destruction of cell populations by electrolytic or chemical lesions causes a decrease in receptors that are located on the dead cells. However, for neurotoxicological studies, this strategy may be of limited value since cell death can often be detected by morphological or neurochemical means more directly and unambiguously.

The remaining mechanism by which alteration of receptor characteristics could occur is as a result of compensatory or adaptive responses of the nervous system to insult or challenge. The issue of response to challenge has been an area of great interest to a variety of neurobiological disciplines during the past decade, and the single greatest emphasis may have been on receptor changes. It is a consequence of these basic studies that a dogma has evolved that is frequently used in neurotoxicology. These ideas may be summarized in general terms, and can then be examined more closely.

Receptor Regulation and Changes in Neuronal Sensitivity. One type of challenge to neuronal systems can be a chronic increase in postsynaptic activation of receptors, for example, from chronic administration of a receptor agonist. This results in a phenomenon called *receptor down regulation* in which, over time, the number of receptors (B_{max}) decreases or, alternatively, the affinity of the receptor for its endogenous ligand decreases (increased K_D). This down regulation would decrease the physiological response resulting from agonist-induced overstimulation, and thus compensate for the chronic administration of drug. The converse situation is also known in which the postsynaptic receptors are chronically deprived of activation. This may be from chronic treatment with a receptor blocking drug, or from damage to the presynaptic nerve terminal, but it results in *receptor up regulation*. This is manifested as an increased number (B_{max}) of receptors or, possibly, as an increased affinity (decreased K_D) of the receptor for its ligand. This would increase the physiological response to the decreased amount of neurotransmitter interacting with the receptor.

There are several consequences of such changes. In the case of "up-regulation," because the number or affinity of receptors has increased, administration of a given dose of an agonist *in vivo* should cause a greater response in the "up-regulated" animal than in control. This shift of the dose-response curve to the left is frequently called *supersensitivity*. Conversely, administration of an agonist to a "down-regulated" animal would cause a decreased response versus control. This shift in the dose response curve to the right is frequently termed *subsensitivity*. We believe that these concepts, as reviewed by numerous authors (cf. 114-116) are the major basis for the widespread use of receptor methods for studying the sequelae of exposure to potential neurotoxicants. Is there a sound foundation for these assumptions?

Denervation studies with skeletal muscle provided an important supersensitivity model. In normally innervated tissue, nicotinic cholinergic receptors are primarily confined to the motor end plate, but denervation results in increased

numbers of cholinergic receptors that are spread more widely over the muscle (117). In the central nervous system, studies of noradrenergic neurons have apparently provided a similar situation. A useful tool for these studies has been 6-hydroxydopamine (6-OHDA), a cytotoxic chemical analog of the endogenous catecholamine neurotransmitters. When administered directly into the brain, the active uptake system for the endogenous neurotransmitters will also imbibe 6-OHDA into the presynaptic nerve terminals. Once inside, 6-OHDA causes the destruction of the nerve terminal, apparently via oxidative mechanisms (118). Using appropriate pharmacological manipulations, it is possible to deplete rather selectively more than 90% of norepinephrine or dopamine in the brain (119). If an adult rat is depleted of norepinephrine in this fashion, permanent compensatory changes are evident. For example, there is norepinephrine-stimulated adenylate cyclase in the cerebral cortex which is sensitive to β-adrenergic agonists such as isoproterenol. After 6-OHDA treatments, the dose response curve for isoproterenol is shifted markedly to the left (i.e., supersensitivity--"up regulation"--has occurred) (120-122). Moreover, the number of β-noradrenergic receptors (estimated using the ligand ^{125}I-iodohydroxybenzylpindolol) is also significantly increased (123).

Studies with 6-OHDA have also provided evidence for subsensitivity changes. Appropriate administration of small doses of 6-OHDA to the postnatally developing rat has two results. Although there is still significant loss of norepinephrine in the cerebral cortex (similar to that described above), the concentration of norepinephrine in the brain stem and cerebellum actually increases. This is the result of sprouting of immature nerve terminals in these regions that occurs because the 6-OHDA-induced insult occurred prior to the cessation of normal neuronal development (124). In these rats, the 6-OHDA-induced increase in norepinephrine content in these caudal brain regions is now accompanied by a decrease in β-receptors (125). Thus, the compensatory change to increased transmitter availability is a subsensitivity.

A final series of examples of up and down regulation in the CNS is particularly relevant. Dopaminergic systems have probably received much attention in this area because of the hypothetical role of this neurotransmitter in several psychiatric and neurological disorders. For example, it is generally accepted that a major action of most clinically useful antipsychotic drugs is their ability to block dopamine receptors (126). Thus, it has been hypothesized that chronic administration of these drugs to humans or laboratory animals will, at withdrawal, cause an increased sensitivity resulting from receptor up regulation. This would be a compensatory response to the receptor blocking effects of the drugs. In

fact, animals treated with and withdrawn from antipsychotic drugs do have consistent increases in putative dopamine receptors, as assessed by radioligand techniques. Moreover, there appears to be the requisite increase in pharmacological sensitivity to dopamine agonists in the same animals (127-128).

The cytotoxic agent 6-OHDA has also been used to study dopamine systems. The animal model most extensively employed involves the injection of 6-OHDA into the substantia nigra, the region in which the dopamine-containing neurons having major efferent fibers to the caudate nucleus are located. If the 6-OHDA lesion is performed on only one side of the brain (the substantia nigra being bilateral), the compensatory changes occur asymmetrically and rats so treated will also demonstrate asymmetrical postural effects when challenged with indirect or direct dopamine agonists (129). From the previously discussed scheme, it is assumed that the side of the brain with the 6-OHDA lesion will become supersensitive, whereas the contralateral, unlesioned, side will not. The available pharmacological data are generally consistent with this idea, and the side with the lesion also has been shown to have increased dopamine-stimulated adenylate cyclase activity as well as increased numbers of dopamine receptors (130-131).

Numerous publications have reported data consistent with these observations, and the views of Reisine (114) are typical of many workers:

> It is apparent from these studies that catecholamine receptors compensate for changes on the level of transmission through alterations in sensitivity. Most of these variations in sensitivity are manifest in either an increase or decrease in receptor density. Since biochemical evidence exists, indicating that catecholamine receptor density can be changed in very short time periods, the phenomenon may be a feasible mechanism to explain the adaptability of such receptors to fluctuations in transmission in the brain.

From this viewpoint the simple concept of receptors up or down regulating in response to perturbations of the nervous system suggests that this would be a valuable tool for neurotoxicologists. However, we propose that the available literature suggests that in many instances other mechanisms are involved in accommodation to toxic insult. Judicious conclusions may well await better understanding of the involved mechanisms.

Studies with opiate systems provide a relevant starting point in support of this latter view, since these systems were the ones in which radioligand methods first were used with CNS tissue. It is known that animals treated for long time periods with opiates (such as morphine) develop tolerance, and if administration of the drug suddenly ceases, there are pronounced withdrawal symptoms. If one regards morphine primarily as an agonist, upon withdrawal from drug treatment it

would be expected that receptor down regulation would occur resulting from chronic receptor occupancy during drug administration. In fact, the symptoms and pharmacology of morphine withdrawal are consistent with this notion. However, receptor changes do not neatly fit this pattern--many investigators report no changes (56,132-135), whereas others find increases (136-139). Although many complicating factors such as receptor subtype or assay conditions affect interpretation (140), it seems illustrative that Pert *et al.* (136) reported that receptor enhancement was evident after a few minutes. Since tolerance, in general, develops over longer time intervals, the simple concept of up-down regulation, as outlined earlier, appears to have limitations.

Is it possible, however, that up and down regulation is valid for classical neurotransmitters such as norepinephrine, dopamine and acetylcholine, but not for the newer chemical messenger candidates that may serve more as neuromodulators? Even this more limited view is probably incorrect. As an example, this laboratory has completed studies using another model of 6-OHDA supersensitivity in which 6-OHDA is given into the cisterna magna. The consequence of this treatment is a dramatic supersensitivity to direct acting dopamine agonists, greater than that seen after unilateral 6-OHDA lesion or after withdrawal from antipsychotic drugs. Yet, this increase in the potency and efficacy of dopaminergic drugs *in vivo* is not paralleled by increases in dopamine receptors using the same techniques applied to the other models (141). With serotonergic systems, a lack of temporal correlation has been shown between alterations in receptors and neuronal adaptation (142).

In summary, we feel that a balanced view of the literature demands conservative use of radioligand methods only in concert with other techniques. The conclusions of Fleming (116) seem to be particularly judicious:

> Postjunctional supersensitivity is a cellular compensatory phenomenon by means of which excitable cells appear to be able to adjust to chronic changes in the stimulus received. It is frequently manifest as a nonspecific increase in irritability. In any one cell, multiple cellular changes (increased density of receptors, altered membrane properties, changes in electrogenic Na^+, K^+ pumping, alterations in Ca^{2+} sequestration, etc.) can contribute to the supersensitivity. Which cellular change(s) is (are) most important varies from one type of effector cell to another.
>
> Although much is known of the cellular mechanisms of supersensitivity in skeletal and smooth muscle, broad opportunities exist for determining the cellular basis of the phenomenon in cardiac muscle, glands and nervous tissue. Many applications of an understanding of supersensitivity to human biology probably remain to be discovered. Finally, although many investigators have searched for the identity of the trophic factor(s) controlling sensitivity,

the evidence is conflicting. The "trigger" may be the cell's ability to recognize its own long-term activity and to adjust to it, the neurotransmitter itself, some other substance from the nerve or a combination of these. This problem still awaits a resolution.

Neurotoxicological Studies Using Radioreceptor Methodology

The use of the radioreceptor assay in neurobiology has been proposed as a means for assessing neurotoxicity (see 106, 108 for review). The purpose of this section is to review available data relating to use of the radioreceptor assay to examine CNS toxicity, and to discuss the relative strengths and weaknesses of the approach. This portion of the chapter will focus on the application of the radioreceptor assay in situations where the effects of *in vivo* administration of the toxicant on subsequent alterations in receptor function were determined. Clearly, advances in radioreceptor assay methodology will enable the neurotoxicologist to examine aspects of CNS toxicity in greater detail and to formulate hypotheses concerning the mode of action of certain neurotoxicants.

Acrylamide. This vinyl monomer is one of the most throughly investigated neurotoxicants with respect to its effects on receptor binding (see 143 for review). Using the radioreceptor screening protocol proposed by Bondy (106), the effects of this compound on dopamine (DA), serotonin (5-HT), acetylcholine (ACh), GABA, glycine and benzodiazepine (BDZ) receptors have been investigated using both developmental and adult dosing models. Gerhart *et al.* (144) administered 75 mg/kg acrylamide to rat pups on day 5 of life, or 25 mg/kg on days 5, 7 and 9 of life. Decreased ^{3}H-spiperone binding in the striatum was seen in the males from the 75 mg/kg group when sacrificed at day 13 or day 21 of life. In a study by Agrawal and Squibb (145), female rats were dosed with 20 mg/kg of acrylamide or vehicle from days 7-17 of gestation. Pups were cross-fostered and sacrificed on either day 14 or day 21 of life. Striatal ^{3}H-spiperone binding (1 nM of radioligand) was decreased in pups sacrificed at two weeks of age from litters where the mother had been treated with acrylamide. This effect was not apparent in pups sacrificed at three weeks of age. Scatchard analysis also revealed decreases in K_D of pups from treated mothers.

It is interesting that the decrease in B_{max} in acrylamide-treated pups corresponds with a lower K_D (higher affinity) in these same animals; it is possible that any physiological changes resulting from a decrease in receptor numbers were offset by a higher affinity of the receptors. Of greater importance, decreases in ^{3}H-spiperone binding were not consistent among groups. In males, decreases occurred in treated pups paired with either a control mother or a treated mother.

But in females, decreases were seen not only in treated pups paired with a treated mother, but also in control pups paired with a treated mother. This suggests that acrylamide had effects that were not fully evaluated in this experimental design.

Agrawal *et al.* (146) treated adult rats with 50, 100 or 200 mg/kg acrylamide. Twenty-four hours after treatment, increased binding was seen in the 100 mg/kg group using either a point-to-point (1 nM ligand) comparison or a Scatchard analysis. The K_D in these latter animals was also decreased. Repeated administration of daily doses of 10, 20 or 30 mg/kg acrylamide for 10 days also increased ^{3}H-spiperone binding 24 hr after dosing. Scatchard analysis of the 10 mg/kg group again showed a decreased K_D and increased B_{max} in treated animals. These effects may have been confounded by decreases in body weight and striatal wet weight that occurred in the treated animals. In both the acute and repeated dosing experiments binding had returned to control values 8 days after dosing. Subsequent studies by this group (147), using single ligand concentrations of a variety of ligands, found increases in ^{3}H-spiperone binding in striatum 24 hr after administration of a single dose of 25, 50 or 100 mg/kg acrylamide. Increases in ^{3}H-strychnine and ^{3}H-5-HT binding were noted only at the highest dose. With repeated administration of 5, 10 or 20 mg/kg acrylamide over a two-week period (148), all doses caused increases in ^{3}H-spiperone and ^{3}H-QNB binding in the striatum 24 hr after the last dose. At this time point increases in ^{3}H-5-HT binding in frontal cortex, ^{3}H-strychnine binding in medulla and ^{3}H-muscimol binding in cerebellum were observed at the highest dose of acrylamide. Decreases in protein content also occurred in striatum 24 hr after dosing for all groups. When animals were sacrificed 8 days after the last dose of acrylamide, increases in ^{3}H-spiperone binding in the striatum were still evident.

These studies also attempted to demonstrate a functional correlate to the increases in receptor number and affinity. Since ^{3}H-spiperone primarily labels dopamine receptors in the striatum, motor activity, a dopamine-mediated behavior, was measured in treated and control rats before and after a 1 mg/kg apomorphine challenge. In animals given both acute and repeated acrylamide dosing, decreases in the activity of treated rats were noted when tested 24 hr after dosing. In animals exposed chronically, this effect was not evident eight days following the last acrylamide administration. The authors interpreted these findings as indicative of destruction of dopaminergic nigrostriatal neurons, resulting in "denervation supersensitivity" (147). As was discussed in the section on Model Systems, damage to nigrostriatal dopamine pathways may cause an increase in striatal dopamine receptors. However, an *increased* response to a pharmacological

challenge that stimulates dopamine receptors (such as apomorphine) would be expected. This suggests that the changes in activity observed in acrylamide-treated rats are not related to an increase in dopamine receptor sensitivity subsequent to nigrostriatal neuronal damage.

A final study (149) attempted to demonstrate that the effect of acrylamide on striatal dopamine receptors was postsynaptic in nature. Rats were lesioned unilaterally with kainic acid, a rigid analog of glutamic acid that is believed to preferentially destroy neuronal perikarya (150). Following recovery from surgery, animals received ten daily doses of either 20 mg/kg acrylamide or the distilled water vehicle. Rats were sacrificed 24 hr after the last dose and binding experiments performed on the lesioned and unlesioned striata using 1 nM ^{3}H-spiperone. Although as in previous work (146,148) binding was increased in unlesioned striata of acrylamide-treated rats, no such difference was observed in the striata of lesioned rats treated with acrylamide. Additionally, no alterations were found in the concentrations of the acidic dopamine metabolites DOPAC and HVA in the acrylamide-treated animals, indicating that acrylamide had no presynaptic effects. The authors concluded that the effect of acrylamide on dopamine receptors as measured by ^{3}H-spiperone binding was postsynaptic in nature. However, kainic acid has also been shown to destroy cholinergic and GABAergic cell bodies in the striatum (151), to alter enzyme activity (152), and to affect neurotransmitter and amino acid content in this brain region (153, 154). These additional actions of kainic acid in the striatum may destroy interneurons and efferent pathways and disrupt the normal neurotransmitter balance in this structure. Thus, interpretation of the data on the effects of acrylamide on dopamine receptors may be confounded by these experimental problems. It is possible that conclusions localizing the effects of acrylamide to the postsynaptic receptor may be premature.

Uphouse and Russell (155) further characterized the changes due to acrylamide by administering a single dose of 100 mg/kg acrylamide and sacrificing rats at 0.5, 2, 4, 6, 8, 10, 12 and 24 hr after dosing. Striatal ^{3}H-spiperone binding was increased at all time points except 6 and 8 hr post-dosing, and forebrain ^{3}H-5-HT binding was also increased from 2-24 hr post-dosing. A final study (156) from this group examined the effects of handling on acrylamide toxicity in rats. A regimen combining pre-treatment handling and administration of 100 mg/kg acrylamide caused decreases in striatal ^{3}H-spiperone binding. However, this same treatment also caused decreases in striatal protein content. A further complication in this experiment is the fact that circadian effects were noted. Rats in the no-handling plus acrylamide group that were sacrificed in the

afternoon had increased binding, and effects of time of sacrifice and time by treatment interactions were significant.

In summary, the available data indicate that acrylamide may preferentially cause changes in striatal ^{3}H-spiperone receptors that are usually reversible after cessation of acrylamide treatment. This increased binding occurs as soon as 0.5 hr post-dosing, and at higher doses of acrylamide also occurs with other ligands. Furthermore, these results may be influenced by handling or time of sacrifice. However, several alternate explanations are possible for the effect of acrylamide treatments on DA receptors. As previously noted, changes in protein content and striatal weight may influence the results. Moreover, in most cases Scatchard analyses were not reported, so it is difficult to determine to what extent decreases in binding at a single ligand concentration are a result of effects on the B_{max} or K_D. Where Scatchard data are reported, decreases in B_{max} and K_D values for ^{3}H-spiperone occurred in two-week old pups exposed to acrylamide prenatally (145) but in adult rats increases in B_{max} and decreases in K_D occurred in both acute and repeated dosing regimens (146). An especially telling point is the inconsistency between the binding data in adult animals and the locomotor activity after apomorphine challenge (147). Finally, due to the rapid onset of the effects on receptors, and the reversibility of these effects, it is possible that acrylamide may cause more generalized effects on membranes and thus influence receptor binding only indirectly. A similar case could be made concerning the effects of acrylamide on protein values. Clearly, further research is necessary to establish more rigorously the role of acrylamide in influencing receptor binding characteristics, as well as to examine possible effects of acrylamide on other dopaminergic systems.

Toxic and Trace Metals. Lead and other heavy metals are compounds that have generated a great deal of toxicological interest. Much of the available data concerning the effects of lead on receptor binding have been collected by Memo, Trabucchi and their collaborators (for review, see 157). In their studies rat dams were given a solution of 2.5 mg/ml lead or sodium acetate in the drinking water. Animals were sacrificed for neurochemical analysis at approximately 5-6 weeks of age. Initially no effects on dopamine receptors were found as measured by Scatchard analysis of ^{3}H-spiperone binding in striatum (158). In a subsequent study (159), Scatchard analysis revealed no differences between lead-treated and control rats in ^{3}H-spiperone binding in either striatum or nucleus accumbens. However, when ^{3}H-sulpiride (a drug purported to be specific for the D_2 dopamine receptor subtype) was used as ligand, Scatchard analysis revealed an increased

B_{max} in the striata of lead-treated animals, whereas the B_{max} was lower in the nucleus accumbens of treated rats. This group also investigated the effects of lead on ^{3}H-GABA binding after administration of either 0.04 mg/ml or 2.5 mg/ml lead (160,161). They reported a decreased B_{max} in striatum and an increased B_{max} in cerebellum, the latter result confirmed by Silbergeld *et al.* (162) who also noted an increase in K_D for ^{3}H-GABA binding in cerebellum.

Our laboratory has also investigated the effects of lead on dopaminergic function as determined by striatal ^{3}H-spiperone binding and by the occurrence of lithium-induced polydipsia (LIP) differences in lead-treated and control rats. When animals are treated with lead by gastric intubation from days 2-29 of life (day of birth = day 0) and tested for the drinking response to repeated lithium injections as adults, rats that received lead as neonates exhibit an exaggerated LIP (163). Subsequent studies have determined that the response to LIP in normal rats is dependent upon an intact nigrostriatal dopaminergic pathway (164). In addition, a single exposure to lead on either day 5 or day 15 of life will result in an increased LIP in adult rats. Although these data suggest that lead treatment in some way alters the responsiveness of the nigrostriatal system to lithium, this alteration is not reflected by changes in striatal ^{3}H-spiperone binding in rats treated with lead at day 5 or day 15 of life (165). When rats are treated with lead from days 2-29 of life, a slight (10%) increase in the B_{max} of striatal DA receptors is evident, but this difference is not significant (DeHaven *et al.*,. unpublished data). However, changes in binding parameters may become evident when rats are sacrificed while still being treated with lithium. Further studies will attempt to resolve this question.

Two reports have been concerned with the effects of manganese on receptor binding. Seth *et al.* (166) administered manganese to adult rats daily for 15 days at a dose of either 10 or 15 mg/kg. Twenty-four hours after the last dose, rats were sacrificed and striata, frontal cortex and cerebellum were dissected. At a dose of 10 mg/kg manganese, only ^{3}H-spiperone binding in striata was elevated. The higher dose of manganese also caused decreases in ^{3}H-QNB binding in striata, ^{3}H-muscimol binding in cerebellum and ^{3}H-5-HT binding in frontal cortex. Similarly, administration of 10, 20 or 40 mg $MnCl_2$/ml of drinking water resulted in increased ^{3}H-spiperone binding in striatum and decreased ^{3}H-muscimol binding in hippocampus (167).

Cadmium has also been reported to affect cholinergic receptor function. When rats received either 1 or 10 mM cadmium chloride ($CdCl_2$) in their drinking water for 8 days and were then sacrificed, binding of 1 nM ^{3}H-QNB was reduced in striatum and cerebral cortex (168). A similar decrease in striatal and cortical

^{3}H-QNB binding was observed in 21-day-old rat pups whose mothers had been given 1 mM $CdCl_2$ in the drinking water (169).

Although these studies demonstrated changes in receptor function, caution must be exercised in interpreting the results. In the Memo and Trabucchi studies (158-161), it is not known which sex was used in binding assays or if a sex effect was present. The dosing regimen did not allow a precise amount of lead to be given to each animal, and no determination of the amount of lead in maternal milk was made. Moreover, the amount of lead in tissue samples from the treated animals is not reported. In addition, since tissue was pooled from each treatment group, failure to observe an effect may be attributable to an increased variability in lead-treated animals. The changes cited after treatments with manganese or cadmium are interesting, but it is not known if they are transient or permanent, especially in the case of neonatal administration. Further research may detect long-term receptor changes due to these metals, as has been suggested for lead.

Cholinesterase Inhibitors and Other Pesticides. Organophosphate pesticides act primarily by inhibiting acetylcholinesterase activity (170). Tolerance to these compounds is known to occur after repeated administration (171). Several laboratories have investigated the effects of organophosphates on the brain muscarinic cholinergic ACh receptor in an attempt to delineate the role of receptor alterations in the development of organophosphate toxicity and tolerance.

Schiller (172) examined the effects of diisopropylfluorophosphate (DFP) on ^{3}H-QNB binding in various brain regions. An initial dose of 1 mg/kg DFP was administered, followed by doses of 0.5 mg/kg every 72 hr. Scatchard analysis revealed decreases in the B_{max} in striatum, hippocampus and cortex of rats sacrificed 24 hr after the last dose of DFP. Ehlert *et al.* (173) treated rats with an initial dose of 2 mg/kg DFP followed by 1 mg/kg on alternate days. Scatchard analysis of ^{3}H-QNB binding found decreases in B_{max} of striata of DFP-treated rats beginning at the first day of DFP treatment and reaching a maximum at 4 days of treatment. This decrease in the B_{max} persisted up to 30 days of DFP treatment. A second experiment found that decreases in binding were not due to the presence of DFP in striatal tissue. Increases in the K_i s of various cholinergic drugs, particularly for agonists, were noted at 10-14 days of treatment, suggesting that DFP may exert its effect at the agonist binding region of the muscarinic ACh receptor. Finally, these researchers demonstrated a correlation between the development of DFP tolerance and the decrease in striatal

^{3}H-QNB binding, suggesting that tolerance to DFP is in part a receptor-mediated phenomenon.

Similar results were obtained in studies with the compound disulfoton (174). Mice received either 7 or 10 mg/kg disulfoton for 14 days and were sacrificed 24 hr after the last dose. The B_{max} for ^{3}H-QNB binding was decreased in forebrain at both doses, and in hindbrain at the 10 mg/kg dose. In a separate experiment where 10 mg/kg disulfoton was given daily and mice were sacrificed at various times during dosing, decreases in ^{3}H-QNB binding did not occur until day 7 in forebrain and day 9 in hindbrain, whereas acetylcholinesterase activity was decreased from day 3 onward. A follow-up to this study (175) using lower doses of disulfoton (initially 2 or 2.5 mg/kg raised to 3 or 3.5 mg/kg over various periods of time) also found decreases in ^{3}H-QNB binding in forebrain and hindbrain that correlated with the appearance of tolerance.

Chlordecone (Kepone®) is another pesticide that causes changes in receptor binding characteristics when administered to both developing and adult rats (176). Chlordecone (1 or 6 ppm) was administered in the diet to female rats from 60 days prior to mating through day 12 post-parturition. Offspring were sacrificed at day 30 of life. Binding of ^{3}H-spiperone (1 nM) was increased in the striata of male offspring of mothers treated with 6 ppm chlordecone. When chlordecone was given to adult male rats at doses of 10 or 30 ppm for 90 days, decreases in binding of ^{3}H-spiperone in striatum and ^{3}H-QNB in cerebellum were seen in rats treated with 10 ppm, and for ^{3}H-muscimol binding in cerebellum of rats treated at both doses. However, striata and cerebelli had increased protein content compared to controls, suggesting that alterations in receptor binding were secondary to the effects of chlordecone on protein synthesis.

A recent preliminary study (177) of the effects of pyrethroid insecticides on mouse forebrain ^{3}H-kainic acid binding showed that the decrease in binding due to incubation with varying concentrations of pyrethroids correlated well with concentrations of these compounds measured in mouse brain at time of onset of toxicity, and with *in vivo* potency when administered intracerebroventricularly. The order of potency was decamethrin > cis-permethrin > trans-permethrin. These researchers concluded that the neurotoxicity of pyrethroid insecticides may involve glutaminergic mechanisms. It remains to be determined if the effects of pyrethroids on ^{3}H-kainic acid binding are solely due to the presence of the insecticide in brain tissue, or if the effects persist after metabolism has occurred.

The available evidence indicates that organophosphates may induce tolerance to their effects by a s sensitivity of the ACh muscarinic receptor. This is

certainly true in the case of DFP and disulfoton and further research may determine if this effect is true for other organophosphates. With chlordecone there appear to be central effects on binding in rats exposed during development, although the effects of chlordecone in adults are less clear. Finally, the excitotoxic effects of the pyrethroid insecticides may be mediated by glutaminergic systems in the brain.

Miscellaneous Compounds. A report by Agrawal *et al.* (178) examined the effects of *in utero* exposure to 3,4,3',4'-tetrachlorobiphenyl (TCB). Pregnant mice were given TCB on days 10-16 of gestation and offspring were sacrificed at approximately one year of age. Binding of 1 nM ^{3}H-spiperone was decreased in TCB-exposed offspring, and this decrease was correlated with the degree of rotational behavior exhibited by the TCB-exposed mice.

A few studies have examined the effects of organometals on receptor binding. Trimethyltin (TMT) did not affect ^{3}H-GABA or ^{3}H-QNB binding *in vitro* (179,180), or ^{3}H-QNB binding in mouse forebrain after *in vivo* administration of 4.26 mg/kg (180). However, Sumner and Hirsch (181) found regional decreases in ^{3}H-QNB binding in both rats and mice. A dose of 2.5 mg/kg TMT in mice produced decreased binding in hippocampus at 4 days, and in amygdala and frontal cortex at 19 days post-administration. When two injections of 2.5 mg/kg TMT were given 5 and 7 weeks before sacrifice, decreases in amygdala ^{3}H-QNB binding occurred. Rats dosed with 7 mg/kg TMT and sacrificed 3.5 weeks later showed decreased binding in amygdala and striatum. Our laboratory (182) found no changes in striatal ^{3}H-spiperone binding 7 days after administration of 7 mg/kg TMT to adult male rats. The toxicity of TMT may be partly due to mechanisms other than receptor changes, although additional systems have not been examined.

Corda and coworkers (183) investigated the effects of a single administration of 10 mg/kg methylmercury on ^{3}H-diazepam binding in rats. Increases in the B_{max} as determined by Scatchard analysis were observed at 3 days post-dosing in cortex, striatum and cerebellum, with the increase in cerebellum persisting up to 10 days after methylmercury exposure. However, no changes in ^{3}H-GABA binding were observed.

A recent report (184) explored the effects of acute and chronic toluene inhalation on ^{3}H-5-HT binding. Rats were sacrificed 3 hr after their final exposure to toluene (either a single 15 min exposure or the same exposure daily for 14 days). Scatchard analysis of ^{3}H-5-HT binding in whole brain revealed a decrease in the B_{max} of chronically treated rats. Regional analysis of binding at a ligand concentration of 4 nM indicated decreases in binding in hippocampus,

and medulla oblongata-pons. Again, since a single ligand concentration was used for the regional studies, any conclusions drawn from this experiment are tentative.

SUMMARY

The studies discussed here illustrate some applications of radioligand methods to neurotoxicological problems. It must be emphasized that before it can be stated that a toxicant affects the binding of radioligands to receptors, other non-specific effects of the compound in question must be ruled out. The degree of lipid solubility of a given compound and its ability to enter the membrane may alter binding characteristics (95). As seen in the case of acrylamide (148, 156) and chlordecone (176), effects on protein content can alter binding values and possibly lead to "false positive" results. This is also true if data are calculated on a per-gram wet tissue weight basis. Additionally, differences due to age, sex, strain, dosing regimen or circadian rhythm fluctuations should be considered.

Before the radioreceptor assay is used as a tool in neurotoxicology, it is important to understand the methodology and limitations of the assay. Several problems arise when a variety of ligands are used in a "screening" type of procedure with only one concentration of each ligand used for binding. By assessing binding at only one ligand concentration it is easier to obtain statistical significance than if several concentrations are used. Conversely, if a toxicant causes an increase in variability, statistical significance may be obscured and a prominent effect may be overlooked. Without a Scatchard analysis it is impossible to assess the effect of a given toxicant on the affinity of receptors. It is also unknown if the toxicant preferentially affects high- or low-affinity binding characteristics. If positive results are seen with more than one ligand when a battery of ligands is used, it may be due to a non-specific effect of the toxicant or to compensatory changes caused by a primary effect in one neurotransmitter system. One may be unable to determine which system is preferentially involved. Thus, standardization of the radioreceptor assay for use in neurotoxicology is inappropriate. The ability to replicate previous findings and to understand basic principles underlying the radioreceptor assay is more crucial. Only assays that are properly performed will yield information pertinent to the action of a compound and the mechanisms by which it produces CNS toxicity.

Future directions in the neurochemical assessment of CNS toxicity should be toward a mechanistic approach to understand how toxicant exposure affects neuronal function. Detailed radioreceptor assay studies using agonist and

antagonist ligands and various competing drugs may elucidate if a compound acts at one conformation or subtype of receptor. However, the radioreceptor assay alone cannot determine the actions of a toxicant. Studies on uptake, chemical messenger synthesis and turnover, and behavioral studies, for example, must accompany radioreceptor assays to give a complete profile of the effects of a toxicant. This integrated approach will permit the greatest chance of successfully determining how a neurotoxicant affects the CNS.

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